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13. ABSTRACT (Maximum 200 Words)
The prospects of stimulating a patient's own immune system as a therapeutic approach to the treatment of cancer in general, and prostate cancer in particular, is intriguing. However, thus far immunotherapeutic approaches to the treatment of cancer (including prostate cancer) in the clinical setting have not been uniformly successful. We are chemically synthesizing molecular conjugates that comprise a Toll-Like Receptor (TLR) ligand covalently linked to a prostate cancer tumor-associated antigen protein or peptide. Using this tactic, we anticipate that the TLR-ligand portion of the conjugates will stimulate dendritic cells (DCs) (as well as other TLR-expressing antigen presenting cells) to secrete immune-activating cytokines. Concomitantly the tumor antigen component of the complex will be processed and presented to activated T cells. In this way, a new and potent immune system stimulation and antigen presentation mechanism aimed at the stimulation of combined CD8+ and CD4+ T-cell responses, along with B-cell activation *via* the innate/adaptive immune response connection, is being developed. We have prepared TLR-4 and TLR-7 ligands and are preparing mouse immunization experiments to test the efficacy of this new approach to cancer immunotherapy.

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A. Introduction

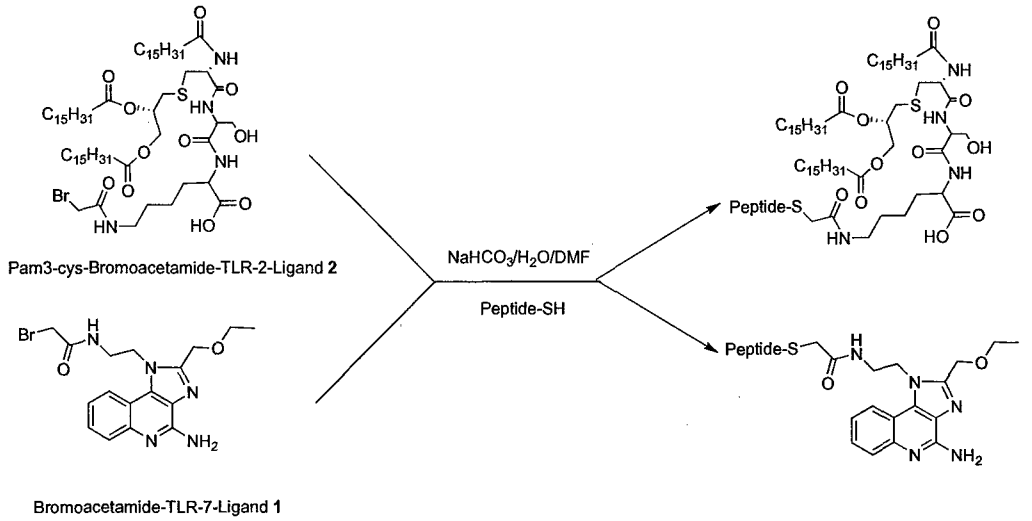
In this project, we are developing a new technique for stimulating a patient's own immune system as a therapeutic approach for the treatment of cancer in general, and prostate cancer, in particular. Thus far, immunotherapeutic protocols for treating cancer (including prostate cancer) in the clinical setting have not been uniformly successful[1]. Many of these methods have been directed toward the stimulation of a CD8+ T-cell response in the host, but have lacked a mechanism for vigorous immune system stimulation and have not combined CD4+ T-cell responses or B-cell involvement[2]. Recently, techniques involving stimulation of dendritic cells (DC) with tumor antigens have begun to show promise, but the process involves isolation of DCs and *in vitro* stimulation, followed by re-injection into the patient. Our methodology uses direct *in vivo* stimulation/maturation of DCs *via* Toll-like receptors (TLRs)[3-5]. We are chemically synthesizing molecular conjugates that comprise a TLR ligand covalently linked to a prostate cancer tumor-associated antigen protein or peptide. Using this tactic, we anticipate that the TLR-ligand portion of the conjugates will stimulate DCs (as well as other TLR-expressing antigen presenting cells) to secrete immune-activating cytokines while the tumor antigen component of the complex will be processed and presented to activated T cells. We have addressed the synthesis of conjugates based on TLR-2 and TLR-7 ligands. The prostate cancer antigen portion of the complexes currently includes an MHC Class I peptide epitope from prostate-specific membrane antigen (PSMA)[6] and a general MHC Class II peptide epitope (PADRE)[7,8]. We have included the complete prostate-specific antigen (PSA) protein linked to a TLR-7 ligand. Our conjugates represent a new and potent immune system stimulation and antigen presentation mechanism with *in vivo* activation of DCs aimed at the stimulation of combined CD8+ and CD4+ T-cell responses along with B-cell activation through the innate/adaptive immune response connection. The conjugates are designed to elicit a vigorous immune response to prostate cancer, and may be administered by s.c. injection, or possibly, nasal or oral routes.

B. Body

This section describes research accomplishments associated with the tasks outlined in the original award application for Year Three of the project.

Task 1. Synthesize lipopeptide carriers covalently conjugated to PSA protein (**months 1-14**)

From previous effort we have completed the synthesis of the antigen conjugates in Figure 1 using the synthetic route outlined in Scheme 1.



Scheme 1. Preparation of peptide conjugates

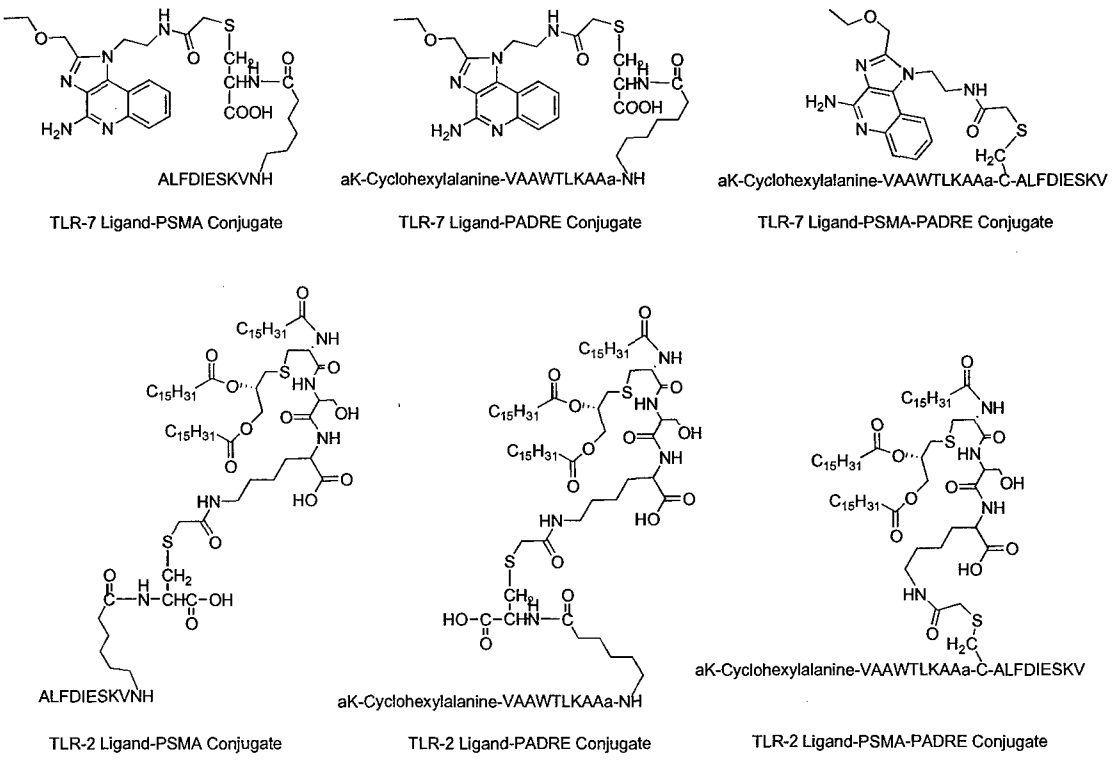
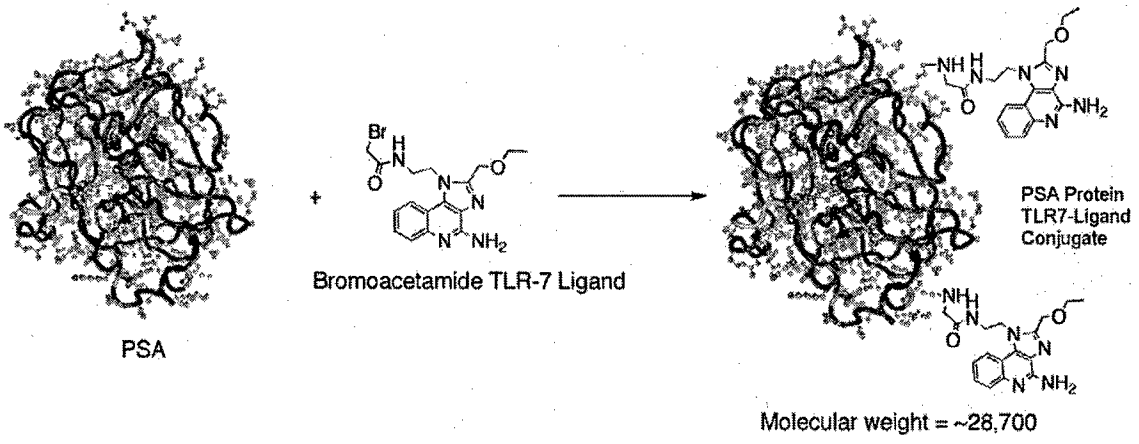


Figure 1. TLR-2 and TLR-7 ligand peptide conjugates

Last time we also reported the preparation of a TLR-7-PSA protein conjugate as outlined

in Scheme 2.



Scheme 2. Preparation of the PSA Protein Conjugate

The PSMA immunostimulatory conjugates are based upon the human PSMA HLA-A2 restricted peptide epitope (ALFDIESKV)[6] and incorporate an aminohexyl spacer between the peptide epitope and a terminal cysteine for attachment to the TLR ligands. The PADRE conjugates comprise the general CD4+ restricted peptide epitope PADRE[7,8] using the identical aminohexyl functionality bonded to a terminal cysteine (aK-clohexylalanine-VAAWTLKAAa-aminohexyl-C). We also synthesized the PSMA epitope covalently linked to the PADRE peptide through a central cysteine (aK-cyclohexylalanine-VAAWTLKAAa-C-ALFDIESKV) in order to evaluate the immunogenicity of conjugates, which contain both MHC Class I and MHC Class II epitopes. The TLR-2 ligand (PAM_3-cys) lipopeptide conjugates proved to be quite insoluble, and thus far we have been unable to either adequately purify the materials or obtain mass spectral characterization data using MALDI, ESI or FAB ionization techniques. Similar difficulties have been noted recently for other PAM_3-cys-peptide conjugates[9]. For this reason, we are using the TLR-7-ligand conjugates of Figure 1 and Scheme 2 in our immunization experiments.

Task 2. Immunize HLA-DR4 and HLA-A2 transgenic mice with lipopeptide-PSA conjugates. Analyze immune responses and test responses against prostate cancer cell-lines (**months 9-25**)

Having prepared immunostimulating conjugates, we began mouse immunization experiments. Founder homozygous mice carrying the HLA-A2.1 Enge transgene that express the human class MHC Class I antigen HLA-A2.1 on cells from the spleen, bone marrow and thymus were obtained from Jackson laboratories, Bar Harbor, ME (Stock Number: 003475). This surface protein recognizes the PSMA peptide epitope that is incorporated into our conjugates. A breeding colony of these mice has been established at The University of Utah. Since we have included the generalized human and mouse MHC Class II antigenic peptide epitope PADRE in our conjugate evaluation, it was not necessary to utilize transgenic mice expressing human HLA-DR4 as originally proposed.

The PADRE peptide is expected to provide the desired CD4+ helper T-cell stimulation. Mice in groups of three were immunized (s.c., axilla) with the TLR-7 ligand PSMA-PADRE conjugate (Figure 2). Three levels of conjugate were initially examined (0.1, 1.0, and 10 μg). Mice were also immunized with the unconjugated PSMA, PADRE, and PSMA-PADRE peptides. Syngenetic C57BL/6J mice that do not express the HLA-A2 gene were used as controls. Twenty-eight days after the initial immunization, each group of mice received a boost vaccination. Eleven days post-boost, splenocytes and peripheral lymph node (PLN) lymphocytes were harvested and [^{3}H]-thymidine cell proliferation assays were performed. Each cell population was administered [^{3}H]-thymidine, stimulated with antigenic peptides (10, 15, 7.5, and 3.8 μg/ml) and controls, and incubated for 24 h. Radioactivity incorporation compared to controls was taken as an indication of the expansion of the cell population in response to antigenic peptide stimulation. Figure 3 summarizes our most recent results. Our previous work used a minimum of 0.1 μg vaccination. We therefore repeated the immunization protocol using a vaccination dose of only 0.01 μg. Figure 2 summarizes these results. It is apparent that even this low amount of antigen also primes lymphocytes in this mouse model. This compares with Figure 4 which shows that even at 1000 times the amount (10 μg) of the PSMA-PADRE peptide (without the covalently attached TLR-7 ligand) elicits no response. This indicates the powerful immunostimulatory effects of the Toll-like receptor-antigen conjugate approach to cancer immunotherapy.

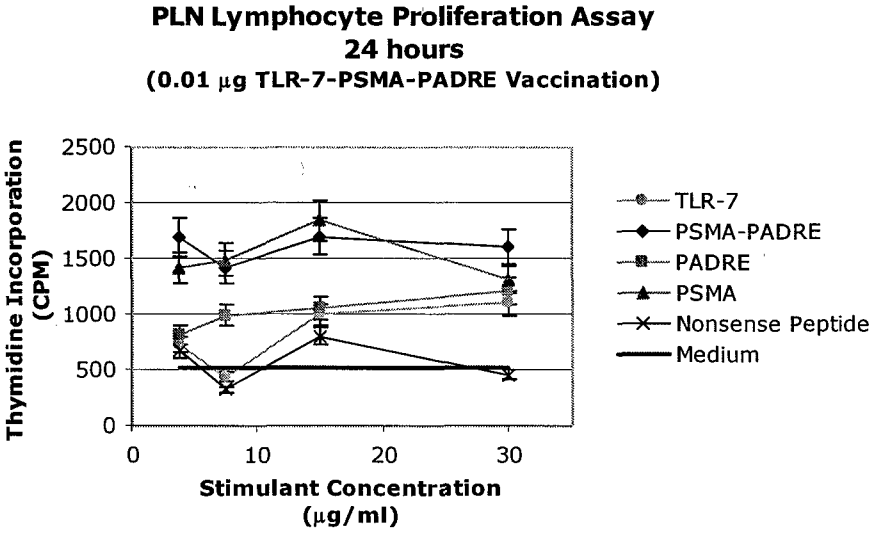


Figure 2. 0.01 μg vaccination. Immune Response

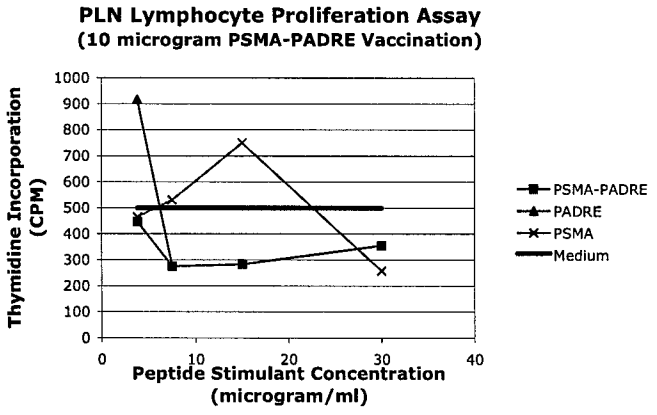


Figure 3. 10 μg PSMA-PADRE Peptide Immunization. No Immune Response

Interestingly, our most recent results with 0.1 μg immunization (Figure 4) show a clear indication of the induction of tolerance to the powerful immune conjugate.

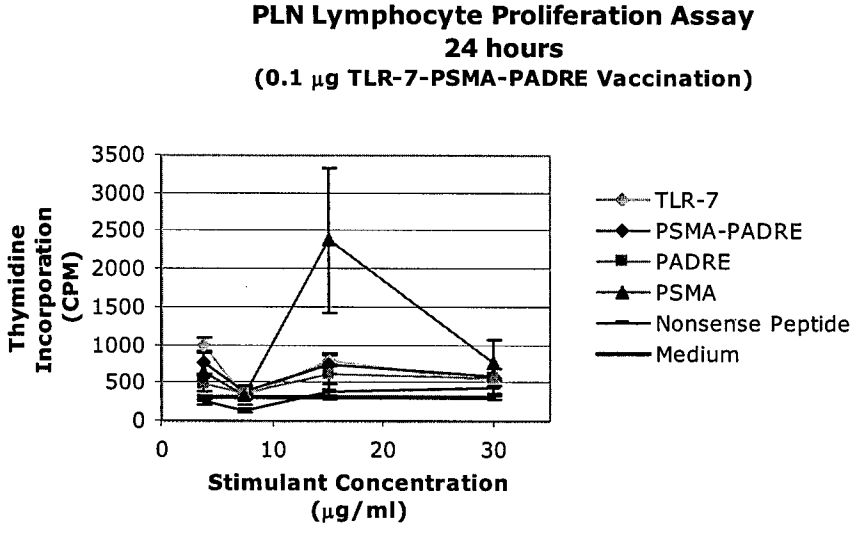


Figure 4. 0.1 μg Immunization. Induction of Tolerance

Figure 5 shows a non-transgenic mouse immunization control.

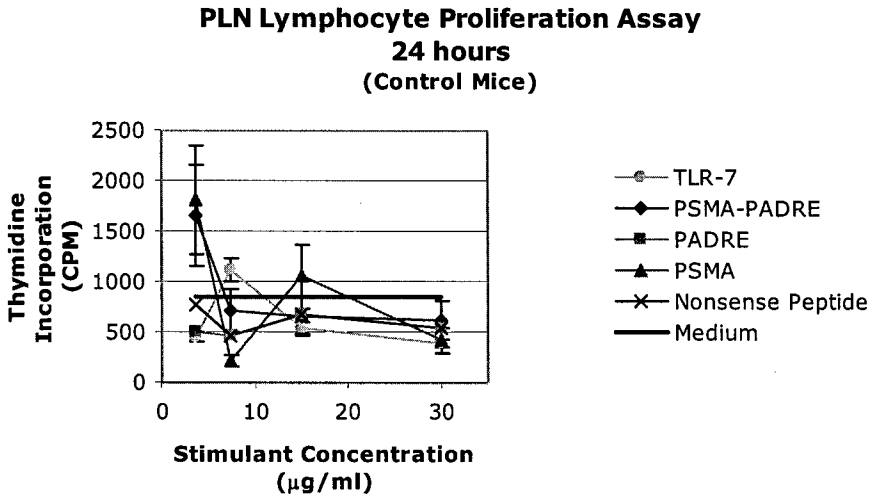


Figure 5. Non-Transgenic Mouse Control. No Immune Response.

Preliminary chromium release experiments evaluating cytotoxic T lymphocyte killing of LNCaP prostate cancer cells (which express the PSMA protein as well as being HLA-A2.1 positive) in culture did not work well at all (Figure 6) in that the response does not smoothly follow the effector to target ratio.

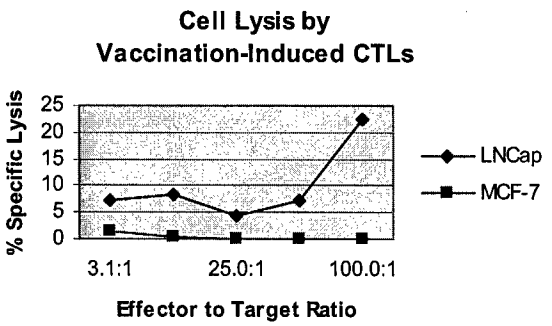


Figure 6. Cell Lysis Experiment. LNCaP Lysis Data Are Not Reliable.

In preparation for these experiments, we experienced difficulty with the uptake and retention of ^{51}Cr by the LNCaP cells using standard labeling protocols. Our first approach to the problem was to obtain different batches of the LNCaP (ATCC) cells. These cells were also found to be difficult to ^{51}Cr label. We then searched for an alternate human prostate cancer cell line that expressed both PSA and PSMA as well as being HLA-A2.1 positive. Several cell lines were evaluated by immunostaining for the necessary markers but we were unable to identify a suitable alternate cell line. We then turned our attention

back to the LNCaP cells. After numerous attempts using multiple cell culture media/conditions (with and without sex hormone supplementation) , we identified circumstances (RPMI with 10% FCS, complemented with non-essential amino acids, sodium pyruvate, mercaptoethanol, and glutamine) which resulted in good cell growth and cells that were readily ^{51}Cr labeled and, importantly, retained the radiolabel. We are now back on track with our immunization experiments and CTL evaluation. Because of these delays, we have requested a no cost extension of the program in order to complete all these originally proposed immunological evaluations.

Task 3. Prepare a PSMA covalent conjugate having a superior lipopeptide carrier determined from **Task 2.** Perform mouse immunization experiments. Test responses against prostate cancer cell-lines (**months 20-36**)

In view of our earlier difficulties with the PSA protein conjugates, we have replaced the PSMA protein-conjugate work with relevant PSMA peptide epitope experiments. These conjugates were synthesized (Figure 1) and their evaluation has been incorporated into **Task 2** which is underway.

C. Key Research Accomplishments

This section provides a list of key accomplishments to date of this research.

- Functionalized ligands recognized by TLR-2 and TLR-7 have been prepared. These ligands were chemically modified with thiol-reactive haloacetamide functionality for attachment of cysteine-containing antigenic proteins and peptides.
- TLR-7 and TLR-2 ligand-antigenic peptide conjugates have been prepared as well as a TLR-7-ligand PSA protein conjugate.
- Mouse immunization experiments are in progress. Data indicate an immunogenic response to the antigenic epitopes.

D. Reportable Outcomes

This program supports graduate research assistant, Mr. Jiang Sha, and the results from his research will be incorporated into his dissertation.

E. Conclusions

Research on this program thus far has provided modified TLR-2 and TLR-7 ligands suitable for chemical attachment of prostate cancer associated antigenic peptides and proteins. The resulting immunostimulating conjugates are being evaluated in mouse immunization experiments in order to ascertain the production of CD8+ and CD4+ T-cells as well as antibodies specific to prostate cancer antigens.

This research is significant in that it represents the first attempt to generate a host immune response to prostate cancer antigens using the potent immune system activation that arises from the innate/adaptive response connection from targeted TLR signaling in combination with antigen presentation. Evaluation of the separate conjugates will provide new insight into the effectiveness of the immune response to the same antigen stimulated by different TLRs. Such effects are not yet known. Furthermore, the possibility of an increased immune response to cancer antigens presented simultaneously with more than one TLR receptor activation path has not been explored. With our newly-synthesized conjugates, we are in a position to initiate these exciting studies.

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G. Appendix

Biosketches

Jerald C. Hinshaw, Principal Investigator

Jiang Sha, Graduate Research Assistant

BIOGRAPHICAL SKETCH	
NAME **HINSHAW, JERALD CLYDE**	POSITION TITLE Research Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing. Include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Oregon State University, Corvallis, Oregon	BS	1962 - 1966	Chemistry
The University of Utah, Salt Lake City, Utah	PhD	1966 - 1970	Organic Chemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: **DO NOT EXCEED TWO PAGES.**

Research and Professional Experience:

1970-1978 Advanced from Senior Research Chemist to Research Associate, Organic Research Laboratory, Chemistry Division, Research Laboratories, Eastman Kodak Company

1978-1984 Scientist, Research and Development Laboratories, Thiokol Corporation

1980, 1986 Member, Utah Award Committee, Salt Lake Section, American Chemical Society

1981 Visiting Research Associate, University of Utah.

1981-1983 Chairman-Elect, Chairman, Past-Chairman, Salt Lake Section, American Chemical Society

1984-1990 Supervisor, Propellant Research Section, Research and Development Laboratories, Thiokol Corporation

1990-1999 Manager, Energetic Materials Research Department, Research and Development Laboratories,Thiokol Propulsion, Brigham City, Utah.

1996-1999 Member, State Advisory Council on Science and Technology (State of Utah, Governor appointment)

1997,1998 Member, Utah State Governor's Medal for Excellence in Science and Technology Award Committee

1997-1999 Chairman, State Advisory Council on Science and Technology (State of Utah, Governor appointment)

1997-1999 Member, Utah Centers of Excellence Program Advisory Council (State of Utah, Governor appointment)

2/99-7/99 Senior Staff to the Technical Director, Science and Engineering, Thiokol Propulsion, Brigham City, Utah

7/99-11/01 Research Assistant Professor, Department of Medicinal Chemistry, The University of Utah, Salt Lake City, Utah

11/01-current Research Associate Professor, Department of Medicinal Chemistry, The University of Utah, Salt Lake City, Utah

Research Interests:

Synthetic chemistry
Synthesis of bacterial oxidosqualene cyclase inhibitors
Cancer immunotherapy
Targeted drugs
Design and synthesis of small molecule inhibitors of protein-protein signaling
Design and synthesis of fluorescent phosphoinositide probes

Research and technology management.

Honors:

Listed in "American Men and Women of Science"
Listed in "Who's Who in Technology"
Named Outstanding Senior in Chemistry, 1966
National Defense Education Act Title IV Fellow, 1968-1970
Franklin Award, Thiokol Corporation recognition for outstanding technical achievement, 1995

Publications/Patents: J. C. Hinshaw has over 50 publications and patents. A few are listed.

P. Y. Lum, C. D. Armour, S. B. Stepaniants, G. Cavet, A. Leonardson, P. Garrett-Engele, M. K. Wolf, L. Butler, C. M. Rush, M. Bard, J. C. Hinshaw, P. Garnier, G. D. Prestwich, G. Schimmack, J. W. Phillips, C. J. Roberts, and D. D. Shoemaker, "Discovering Novel Modes of Action for Therapeutic Compounds using a Genome-wide Screen of Yeast Heterozygotes," *Cell,* 2004, **116**, 121-137.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.

NAME	POSITION TITLE
SHA, JIANG	Graduate Research Assistant

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Peking University, Beijing, China	B.S.	1997-2001	Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Research and Professional Experience:

2000-2001 Institution of Biophysics, Chinese Academy of Science, Bachelors' degree research

2001-2002 Molecular Biology Program, The University of Utah, Laboratory Rotation

2002-current Graduate Research Assistant, Department of Medicinal Chemistry, The University of Utah, Salt Lake City